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(21) International Application Number: PCT/US98/07192 (22) International Filing Date: 8 April 1998 (08.04.98) (30) Priority Data: 60/043,119 9 April 1997 (09.04.97) US (71) Applicant (for all designated States except US): MICHIGAN STATE UNIVERSITY [US/US]; 238 Administration Building, East Lansing, MI 48824 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ZEIKUS, Joseph, G. [US/US]; 2529 Dustin, Okemos, MI 48864 (US). VIEILLE, Claire [US/US]; 2715 Fernwood, Lansing, MI 48912 (US). SAVCHENKO, Alexei [US/US]; 1312i University Village, East Lansing, MI 48023 (US). DONG, Guoqing [CN/CA]; 195 DaFoe Road, Winnipeg, Manitoba R3T 2M9 (CA). (74) Agent: BAKER, Jean, C.; Quarles & Brady, 411 East Wisconsin Avenue, Milwaukee, WI 53202-4497 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: HYPERTHERMOSTABLE α -AMYLASE (57) Abstract A preparation of <i>Pyrococcus furiosus</i> extracellular α -amylase is disclosed. In one embodiment, this α -amylase has an optimal temperature of 100 °C, a pH optimum of 5.5–6.0, and a half-life at 98 °C of between 12 and 14 hours. In another embodiment, the present invention is a gene construct encoding an extracellular α -amylase with an optimum temperature of 100 °C.		

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HYPERTHERMOSTABLE α -AMYLASE

TECHNICAL FIELD

In general, the field of the present invention is starch-hydrolyzing enzymes. Specifically, the field of the present invention is α -amylase enzymes.

5

BACKGROUND ART

α -Amylases (EC 3. 2. 1. 1) are endo-acting enzymes that hydrolyze starch by cleaving α -1,4-glucosidic linkages at random. They are among the most important commercial enzymes having wide applications in starch
10 processing, brewing and alcohol production, textile, and other industries. Numerous α -amylases have been characterized and their genes cloned from eubacteria, fungi, plants, and animals. With the exception of one eubacterial and one archaeal enzymes (18), they all
15 belong to a same α -Amylase family, sharing a similar structure, similar catalytic site, and same catalytic mechanism (22).

α -Amylases contain three domains: (i) domain A corresponds to an $(\alpha_1/\beta)_8$ barrel; (ii) in this barrel, the
20 $\beta_3 \rightarrow \alpha_3$ loop is very long and represents a second domain, domain B; Domain C is a separate globular domain composed of β -strands arranged in a Greek key motif (9). Four highly conserved regions come together through the interaction of domains A and B to form the active center, substrate binding site, and a Ca^{2+} binding site. The Ca^{2+}
25 cation is essential for enzyme folding (9), optimal activity, and stability (43).

Since starch starts being soluble only at 100°C and above, the majority of α -amylase industrial applications
30 require their use at temperatures up to 110°C (17). The most thermostable α -amylase (TAKA-THERM α -amylase) used in industry was purified from *Bacillus licheniformis*. It has an optimal temperature of 90°C and requires additional Ca^{2+} for its thermostability (43).

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Hyperthermophilic archaea are attracting increasing applied research attention since their enzymes show extreme thermostability (2; 42). Many hyperthermophiles can grow on starch and other carbohydrates, suggesting that they express a variety of amylolytic enzymes that could be of industrial interest (1; 42).

Recently, several hyperthermostable amylolytic enzymes have been reported from *Pyrococcus furiosus* (7; 15; 23; 25), *P. woesei* (24) and *Thermococcus profundus* (11). Only the *P. furiosus* intracellular α -amylase gene was cloned and expressed in *E. coli* (26). Its sequence shared very low homology with other α -amylase sequences and did not display any of the four consensus regions.

Needed in the art of starch hydrolysis is an α -amylase with improved thermostable characteristics.

DISCLOSURE OF THE INVENTION

In one embodiment, the present invention is an α -amylase enzyme with an optimal activity temperature of 100°C. Preferably, the enzyme has a pH optimal of 5.5-6.0 and a half-life at 98°C of 12-14 hours.

In another embodiment, the present invention is a preparation of *Pyrococcus furiosus* extracellular α -amylase. In a preferred embodiment, the preparation has an optimal activity temperature of 100°C, an optimum pH of 5.5-6.0, and a half-life at 98°C of between 12 and 14 hours.

In one embodiment of the present invention, the amino acid sequence of the α -amylase comprises SEQ ID NO:1. In another embodiment, the amino acid sequence of the α -amylase comprises residues 27-460 of SEQ ID NO:1.

In another embodiment, the present invention is a gene sequence encoding the α -amylase described above. In one embodiment, the gene sequence comprises SEQ ID NO:2. In a more preferred embodiment, the gene sequence comprises residues 118-1497 of SEQ ID NO:2.

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The present invention is also a method of hydrolyzing starch, comprising the step of mixing starch and the α -amylase described above under conditions suitable for enzyme activity, wherein starch is
5 hydrolyzed into oligosaccharides, preferably G_2 - G_7 .

It is an advantage of the present invention that an enzyme is provided that is suitable for starch hydrolysis at temperatures greater than or equal to 100°C.

Other advantages, features and objects of the
10 present invention will become apparent to one skilled in the art after evaluation of the specification, claims and drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 is the nucleotide sequence (SEQ ID NO:2) and
15 deduced amino acid sequence (SEQ ID NO:1) of the *P. furiosus* extracellular α -amylase gene.

Fig. 2 is a sequence alignment of *P. furiosus* (Pfu) (SEQ ID NO:1) and *B. licheniformis* (Bli) (SEQ ID NO:3) extracellular α -amylases.

20 Fig. 3A, B, and C illustrate *P. furiosus* extracellular α -amylase behavior on 12% polyacrylamide native gel (A), SDS gel (B), and starch-containing SDS gel (C).

Fig. 4 demonstrates TAKA-THERM α -amylase behavior on
25 (A) 12% SDS-PAGE and (B) starch-containing 12% SDS-PAGE.

Fig. 5 is a graph of the effect of temperature on the activities of *P. furiosus* extracellular α -amylase (●) and TAKA-THERM (■).

Fig. 6 illustrates the influence of pH on *P. furiosus* α -amylase (●) and TAKA-THERM (○) activities.
30

Fig. 7 is a graph of thermostabilities of the recombinant *P. furiosus* extracellular α -amylase (■, ●) and TAKA-THERM (x, □) in the absence (■, ●, □) or presence (x) of 5 mM Ca^{2+} at 90°C (x, □, ■) or 98°C (●).

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BEST MODES FOR CARRYING OUT THE INVENTION

The present invention is a preparation of α -amylase enzyme, wherein the enzyme has an optimal activity temperature of 100°C. Preferably, this enzyme is
5 isolated from *Pyrococcus furiosus* and, most preferably, has the amino acid sequence of SEQ ID NO:1.

The Examples below and Dong, et al. (Applied and Environmental Microbiology, 63[9]:3569-3576, September 1997, hereby incorporated by reference) describe the
10 purification of the α -amylase of the present invention from *Pyrococcus furiosus*. Applicants envision that the α -amylase of the present invention may be purified from other hyperthermostable organisms.

The present invention is also a method of producing
15 the α -amylase described above. Most preferably, the method would involve using a DNA sequence encoding the enzyme, such as that described in SEQ ID NO:2, in a genetic construct to express the protein in a host organism.

20 The DNA sequence encoding the enzyme may also be created by genetic engineering methods known to one of skill in the art. For example, applicants have provided a sequence for the cloned gene from *Pyrococcus furiosus*, and one may use this sequence to create primers capable
25 of amplifying the gene from *Pyrococcus furiosus* genomic DNA. Once one has recloned the gene from *Pyrococcus furiosus*, one may place this gene in an appropriate expression vector and express the α -amylase protein in an appropriate host organism.

30 One of skill in the art would realize that SEQ ID NO:2 is not the only nucleotide sequence that could encode an α -amylase of the present invention. One may make modifications, deletions and additions to this sequence and still encode a functionally equivalent
35 protein. (By "functionally equivalent, we mean an α -amylase enzyme meeting the criteria defined below at (1),

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(2) and (4).) For example, the region upstream of residue 118 and downstream of residue 1497 may be deleted because this region does not encode the α -amylase protein. Preferably, the DNA sequence of the present invention encodes the protein sequence of SEQ ID NO:1 or
5 SEQ ID NO:1 with conservative functionally equivalent substitutions.

The enzyme of the present invention can be defined by the following characteristics:

10 (1) The enzyme of the present invention has an optimal temperature of between 95 and 105°C. Preferably, the optimal temperature is 100°C. The Examples below and Dong, *et al.* (*supra*) describe the determination of maximal enzymatic activity of an α -amylase by performing
15 standard enzyme assays at different temperatures. One would examine a candidate enzyme in the same manner.

(2) The enzyme of the present invention has a half-life at 98°C of 12 to 14 hours, preferably 13 hours. The Examples below and Dong, *et al.* (*supra*) describe methods
20 of determining half-life.

(3) Preferably, the enzyme of the present invention has an amino acid sequence of SEQ ID NO:1. However, Applicants envision that conservative or non-conservative substitutions and deletions or additions to SEQ ID NO:1
25 could result in a functionally equivalent enzyme.

(4) The enzyme of the present invention preferably has a pH optimal of 5.5-6.0. The Examples below and Dong, *et al.* (*supra*) describe techniques for determining optimal pH.

30 By "preparation" we mean an enzyme purification whereby the α -amylase is substantially purified from its host organism. A preparation will have a specific activity of at least 350 μ /mg at 98°C. Preferably, a purified enzyme would have a specific activity of at
35 least ~3,500 U/mg at 98°C.

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EXAMPLES

The Examples report the characterization of a hyperthermostable *P. furiosus* extracellular α -amylase. Its gene was cloned and expressed in *E. coli* and its
5 sequence was determined. The recombinant enzyme was purified and characterized. Its catalytic and stability properties were compared to those of the commercial *B. licheniformis* α -amylase (TAKA-THERM).

1. Materials and Methods10 *Bacterial strains and growth conditions*

P. furiosus DSM 3638 was cultivated as described previously (15) at $90^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under anaerobic conditions. Cells were harvested in the stationary growth phase and stored at -20°C before use. *Escherichia coli* Sure strain
15 (Stratagene, La Jolla, CA) was used as plasmid host and grown in LB medium at 37°C . Ampicillin ($100 \mu\text{g/ml}$) was added when required. Plasmid pUC18 (Pharmacia Piscataway, NJ) was used as cloning and sequencing vector.

20 *Library construction and screening*

P. furiosus chromosomal DNA was prepared as reported (3). The chromosomal DNA was partially digested with restriction enzyme *Sau3A*. Plasmid pUC18 cut with *Bam*HI and dephosphorylated was ligated with the 4-8 kb DNA
25 fragments isolated by a 10-40% sucrose gradient. The ligation mixture was transformed into *E. coli* Sure by electroporation and the bacteria were plated on 1.5% agar LB ampicillin plates. After 16-20 hours incubation at 37°C , colonies were replicated onto a new set of LB
30 ampicillin plates containing 1% phytigel instead of agar and 0.2% soluble starch. After overnight growth, the plates were incubated at 80°C for 8-10 hours. Amylase activity was detected by flooding the plates with I_2/KI .

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Nucleotide sequence determination

Restriction analysis and plasmid DNA purification were performed as reported (3). Nested deletions for sequencing were generated on both sides of the insert according to Henikoff (20). Sequences were analyzed in both directions by the dideoxy chain termination technique (36) using Sequenase version 2.0 T7 DNA polymerase sequencing kit (U. S. Biochemicals, Cleveland, OH) and ThermoSequenase kit (Amersham Life Science, Arlington Heights, IL). Sequencing data were analyzed using the GCG Sequencing Analysis Software Package V 7.0 (13). The amino acid sequence of *P. furiosus* extracellular α -amylase was compared with other amylolytic enzymes available through the GenBank/EMBL Data Bank (IntelliGenetics Inc., Mountain View, CA). Two pairs of oligonucleotides (pair 1: 5'-CAAATGTCACGTTGT ATGG-3', SEQ ID NO:4 and 5'-GAGAGTGGTGCAAAGGTC-3', SEQ ID NO:5 and pair 2: 5'-CTGGTGGTGACCTAGAATG-3', SEQ ID NO:6 and 5'-TATCTGTGTCATGATTGGC-3', SEQ ID NO:7) identical to different sequences of the pS4 insert were synthesized and used as primers for PCR with *P. furiosus* genomic DNA as template. The PCR products were analyzed on agarose gels and sequenced. Oligonucleotides were synthesized by the Michigan State University Macromolecular Facility.

Nucleotide sequence accession number

The GenBank accession number for the sequence published in this paper is AF001268.

Site-directed mutagenesis

The initiation codon (GTG) of the *P. furiosus* extracellular α -amylase gene was changed to ATG using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) with primers 5'-GAGGTGATCACATGAACATAAAG AAATIAACACC-3' (SEQ ID NO:8) and 5'-GGTGTTAATTTCTTTAT GTTCATGTGATCACCT-3' (SEQ ID NO:9).

Enzyme purification

All purification steps were performed at room temperature under aerobic conditions. When expressed in *E. coli*, the recombinant *P. furiosus* extracellular α -amylase was not secreted into the medium. Cells carrying the recombinant plasmid pS4 were grown in LB ampicillin. Cell homogenate was prepared by passing through a French press cell at 15,000 lb/inch². After heat treatment at 80°C for 15 min, the cell homogenate was centrifuged at 16,300 x g for 20 minutes. The enzyme was precipitated by adding 60% (NH₄)₂SO₄ to the supernatant, and the pellet was resuspended in 50 mM sodium acetate buffer at pH 6.0.

The concentrated crude enzyme was loaded onto a Phenyl-Sepharose (Pharmacia Fine Chemica AB, Uppsala, Sweden) column (1.5 x 18 cm) equilibrated with 50 mM acetate buffer (pH 6.0). The column was washed with the same buffer, then with 50 mM Tris-HCl (pH 8.0). The enzyme was eluted with 6 M urea in 20 mM Tris-HCl (pH 9.4). After concentration in an ultrafiltration cell equipped with a 30,000 molecular weight cut-off membrane (Amicon, Beverly, MA) and dialysis against 50 mM Tris-HCl (pH 6.0), the enzyme was loaded onto a Phenyl-Sepharose column (1.5 x 18 cm) equilibrated with 50 mM Tris-HCl (pH 6.0). The column was washed with 50 mM Tris-HCl at pH 6.0, then at pH 8.0, and pH 9.4. Finally, the enzyme was eluted with 6 M urea in 20 mM Tris-HCl (pH 9.4). The fractions with α -amylase activity were pooled and concentrated by ultrafiltration (see above).

The concentrated enzyme was loaded onto a Sephacryl S200 (Pharmacia Fine Chemica AB, Uppsala, Sweden) column (1.5 x 80 cm) equilibrated with 20 mM Tris-HCl buffer (pH 9.4) containing 5% glycerol. The active fractions were concentrated by ultrafiltration (see above) and dialyzed against 50 mM sodium acetate buffer (pH 5.6) (buffer A). Commercial TAKA-THERM L-340 α -amylase (TAKA-THERM) from *B. licheniformis*, a gift from Genencor International Inc.

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(Rochester, NY), was dialyzed against 50 mM sodium acetate buffer (pH 6.0).

The NH-terminus of the recombinant *P. furiosus* α -amylase was sequenced by the Michigan State University
5 Macromolecular Facility.

Enzyme assays

P. furiosus extracellular α -amylase activity was determined by measuring the amount of reducing sugar released during enzymatic hydrolysis of 1% soluble starch
10 in buffer A at 98°C for 15 minutes. A control without enzyme was used. The amount of reducing sugar was measured by a modified dinitro salicylic acid method (5). One unit of amylase activity was defined as the amount of enzyme that released 1 μ mol of reducing sugar as glucose
15 per minute under the assay conditions. TAKA-THERM was assayed at 90°C in 50 mM sodium acetate buffer (pH 6.0) containing 0.5 mM Ca^{2+} (buffer B). Other conditions were the same as above. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond,
20 CA) with bovine serum albumin as standard.

Molecular mass determination

A 0.5 x 45 cm column containing Sephacryl S200 was equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl. The purified sample and marker
25 proteins (i.e., carbonic anhydrase [29,000], bovine serum albumin [66,000] alcohol dehydrogenase [150,000], and Blue Dextran [2,000,000] were applied to the column at the flow rate of 7 ml/h. Elutions of the marker proteins and the recombinant *P. furiosus* extracellular α -amylase
30 were followed by 280 nm-UV detection and activity assay.

Gel-electrophoresis

Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 12% polyacrylamide (27). Protein samples were

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denatured by incubation with denaturing buffer (2% SDS and 0.64 M mercaptoethanol, final concentrations) at different temperatures in water or silicon oil baths for varying periods. Low molecular weight protein markers (Bio-Rad, Richmond, CA) were used as standards. Native polyacrylamide gel electrophoresis was performed in the same conditions as above except for the absence of SDS in the buffer system and in gel. Samples were heat treated in 0.1 M acetate buffer (pH 5.6) at different temperatures before loading. Proteins were stained by Coomassie Brilliant Blue R-250. For activity staining, 0.66% soluble starch was added during SDS-polyacrylamide gel preparation. After electrophoresis, starch-containing gels were washed with buffer A and incubated at 90°C for 10 minutes in buffer A. Enzyme activity was visualized by flooding with a I₂/KI solution. Gels containing TAKA-THERM were washed with buffer B and incubated at 80°C for 10 minutes in buffer B. Other conditions were the same as above.

20 *Analysis of hydrolysis products*

The recombinant α -amylase (2.5 U/ml) was incubated at 90°C with 1% (wt/vol) soluble starch, pullulan, glycogen, amylose, amylopectin, or oligosaccharides. Samples were withdrawn after varying periods. Hydrolysis products were analyzed by high-performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) and CarboPac PAI column (4 mm x 250 mm) (Dionex system). Hydrolysis products were identified and quantified using the PEAK II computer software (SRI Instruments, Torrance, CA). Glucose (G₁), maltose (G₂), maltotriose (G₃), maltotetraose (G₄), maltopentaose (G₅), maltohexaose (G₆), and maltoheptaose (G₇) were the standards. Starch, pullulan, glycogen, amylose, amylopectin, and oligosaccharides were also incubated with TAKA-THERM (2.5 U/ml) at 80°C in buffer B, and hydrolysis products were analyzed for comparison.

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pH and temperature studies

The optimal pH for *P. furiosus* extracellular α -amylase activity was determined at 98°C in 50 mM acetate buffer (pH 3.5-4.6) and 50 mM Tris-HCl buffer (pH 6-11).

5 All pHs were adjusted at room temperature and corresponding pHs at high temperatures were calculated using $\Delta pK_a/\Delta T^\circ C = 0.000$ and -0.031 for acetate and Tris, respectively (30). TAKA-THERM was assayed at 90°C in the same buffers in the presence of 0.5 mM Ca^{2+} .

10 The temperatures of maximal activity of *P. furiosus* α -amylase and TAKA-THERM were determined by performing standard enzyme assays at different temperatures.

For stability studies at high temperatures, both enzymes were EDTA-treated. They were first dialyzed
15 extensively against buffer A (*P. furiosus* α -amylase) or buffer B without Ca^{2+} (TAKA-THERM) containing 2 mM EDTA, then twice against the same buffers without EDTA. Enzyme thermal inactivation studies were performed by incubating 1 ml Gas Chromatography tubes (Alltech Associates,
20 Deerfield, IL) that contained 800 μ l purified enzyme in 0.1 M acetate buffer (pH 5.6 for the *P. furiosus* α -amylase and pH 6.0 for TAKA-THERM) in the presence or absence of 5 mM Ca^{2+} at 90°C or 98°C. After various incubation periods, samples were withdrawn and tested for
25 residual α -amylase activity under each enzyme's standard assay conditions.

 α -amylase overexpression in E. coli

The *P. furiosus* α -amylase gene was amplified by PCR without its signal peptide. Oligonucleotide

30 5'-AGCTAGCTTGGAGCTTGAAGAGGGAG-3' (SEQ ID NO:10) was used as forward primer. Sequence AAATCA encoding the two N-terminal residues Lys-Tyr was substituted by GCTAGC, encoding Ala-Ser and creating an *NheI* site.

Oligonucleotide 5'-ACTCGAGACCACAATAACTCCATACGGAG-3' (SEQ
35 ID NO:11) was used as reverse primer. Sequence GITGGG (SEQ ID NO:12) encoding the C-terminal residues Val-Gly

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was substituted by CTCGAG (SEQ ID NO:13), encoding Leu-Glu and creating a XhoI site. The amplified gene was cloned in pCR2.1 (Invitrogen, Carlsbad, CA) and its sequence was verified. It was then subcloned in pET21
5 (Novagen, Madison, WI), yielding recombinant plasmid pET213. In this construct, *P. furiosus* α -amylase is expressed with a (His)₆ C-terminal tail that allows its purification by Ni-NTA resin affinity chromatography. Recombinant plasmid pET213 was transformed in *E. coli*
10 BL21(DE3) (Novagen, Madison, WI). BL21(DE3)(pE1213) was grown in LB medium to the end of the exponential phase and T7 RNA polymerase-dependent expression was induced by isopropyl- β -D-thiogalactopyranoside (IPTG) (6 mM final concentration). After three hours induction, cells
15 extracts were prepared and analyzed by SDS-PAGE and activity assay.

2. Results

Cloning and sequencing of the gene encoding P. furiosus extracellular α -amylase

20 Among about 10,000 clones screened on starch-containing plates, two colonies developed a clear halo. Both transformants expressed thermostable α -amylase activity but no pullulanase activity. They both constitutively expressed the α -amylase in a starch-free
25 medium in the absence of isopropyl- β -D-thiogalactopyranoside (IPTG), the inducer of the lac promoter. The two recombinant plasmids were shown by restriction analysis to contain overlapping inserts. The smallest, pS4, carried a 2.7 kb insert, and was selected
30 for further studies. Plasmid pS4's insert was sequenced entirely. To confirm that the insert corresponded to *P. furiosus* genomic DNA, two pairs of oligonucleotides corresponding to different pS4 sequences were used as primers in PCR reactions. With *P. furiosus* genomic DNA
35 as template, the sequences of the PCR products were identical to the corresponding sequences in the pS4

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insert, indicating that the insert did, indeed, come from *P. furiosus* chromosomal DNA. The pS4 insert contained a single complete open reading frame (ORF1) (Fig. 1).

Fig. 1 is the nucleotide sequence and deduced amino acid sequence of the *P. furiosus* extracellular α -amylase gene. Referring to Fig. 1, the NH-terminus of the recombinant protein is underlined with asterisks. The signal peptide is in italics. Putative promoter sequence, ribosome binding site (RBS), initiation codon, and transcription termination sequence are in bold. The -35 and -10 regions potentially recognized as *E. coli* promoters are underlined. The four conserved regions are framed and numbered.

The 491-residues polypeptide encoded by ORF1 showed an overall 35.7% identity to *B. licheniformis* α -amylase (Genbank accession no. m38570), indicating that ORF1 encoded the α -amylase expressed by pS4. The N-terminal sequence of the *P. furiosus* recombinant α -amylase was determined. It was identical to residues KYLEL located 58-62 residues downstream of ORF1's first ATG (Fig. 1).

Since most α -amylases, including the *B. licheniformis* enzyme, are extracellular enzymes, we checked if ORF1's 57 first residues could correspond to a signal peptide. The 26-residues stretch located just upstream of the KYLEL sequence (Fig. 1) showed all the characteristics of a prokaryotic signal peptide (Watson, 1984). It showed 66.7% and 57.7% similarity to *P. furiosus* (15) amylopullulanase and pyrolysin (46) signal peptides. This 26-residues sequence starts with a valine encoded by GTG (Fig. 1).

At least two *Pyrococcus* genes, the genes for *P. furiosus* intracellular α -amylase (26) and *P. woesei* glyceraldehyde-3 phosphate dehydrogenase (48), have been shown to have a GTG start codon. Several arguments tend to confirm that this new *P. furiosus* α -amylase gene also starts with GTG. (i) The similarity with *B. licheniformis* α -amylase clearly starts at the level of

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this residue, at the N-terminus of the *B. licheniformis* α -amylase signal peptide (Fig. 2). (ii) The sequence GGAGGT located 5 nt upstream of the "starting GTG" (Fig. 1) represents a putative ribosome binding site (RBS).
5 This sequence is identical to the *P. furiosus* maltose-regulated (34) *mlrA* and pyrolysin (46) genes's RBS's. No putative RBS could be identified upstream of any of the two ATG's preceding the "starting GTG". (iii) 52 nt upstream of the "starting GTG" (but downstream of
10 the two ATG's) the sequence TTTATA (Fig. 1) is identical to the consensus defined as box A in archaeal promoters (19). (iv) Since GTG is rarely used as starting codon in *E. coli* genes, the "starting GTG" was mutagenized into ATG. Expression of the mutant enzyme in *E. coli*
15 increased eight times compared to the wild-type enzyme (not shown). All these evidences showed that ORF1 encoded a *P. furiosus* extracellular α -amylase containing a 26-residues signal peptide. ORF1 stop codon was immediately followed by a 19-residues stretch of
20 pyrimidines containing the sequence TTTTCT typical of archaeal transcription termination signals (33). Two truncated ORF's (not shown but in Genbank) were detected upstream and downstream of ORF1, in the opposite orientation. Neither of them showed significant homology
25 to any sequence present in the GenEMBL database.

The G+C content of this new *P. furiosus* α -amylase gene was 41.9%, slightly higher than the value (38%) reported for the total genome (16). As has been seen in other genes sequenced from hyperthermophiles, A and T
30 were the preferred bases (62%) in the third position of codons (48). Proline and threonine codons ending with G were rarely used. Like other reported hyperthermophilic archaeal protein genes (39), AGG and AGA arginine codons were strongly preferred. Interestingly, *P. furiosus*
35 extracellular α -amylase contained five cysteines.

Archaeal genes can generally not be directly expressed from their own promoters in *E. coli*. The two

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sequences TTCACA N17 TTATAT and TTTATA N17 TACATT located 80-52 and 58-29 nt upstream of the GTG start codon, respectively, are close to the *E. coli* consensus promoter sequence. One of them is probably responsible for *P. furiosus* α -amylase gene expression in *E. coli*.

Comparison of P. furiosus and B. licheniformis α -amylase sequences

P. furiosus extracellular α -amylase showed 45-56% similarity and 20-35% identity to eubacterial α -amylases and other enzymes of the α -amylase family (e.g., neopullulanase, pullulanase, isoamylase, amylopullulanase) (not shown). The closest enzyme was *B. licheniformis* α -amylase (55.7% similarity and 35.7% identity) (Fig. 2).

Fig. 2 is a sequence alignment of *P. furiosus* (Pfu) (SEQ ID NO:1) and *B. licheniformis* (Bli) (SEQ ID NO:3) extracellular α -amylases. Vertical lines and columns denote identical and similar residues, respectively. Numbering starts after the signal peptides. Signal peptides are in italics. The four active site conserved regions are in bold. (α/β) Barrel (domain A) and domain B are framed and shadowed, respectively. Underlined sequences correspond to the secondary structures in the *B. licheniformis* α -amylase (α/β) barrel, as described by Machius, et al. (28). Corresponding secondary structures are indicated under the sequence. *B. licheniformis* α -amylase residues involved in Ca^{2+} (*) and Cl (●) (28) are indicated under and above the sequence, respectively.

Conservation was not uniform along the whole sequence. In particular, the sequences corresponding to the (α/β) barrel domains (or domains A), domains B, and domains C showed 40%, 22%, and 30% identity, respectively. *P. furiosus* α -amylase is 10% shorter than the *B. licheniformis* enzyme. *P. furiosus* α -amylase domains A, B, and C were 9%, 29%, and 17% shorter, respectively, than the corresponding domains in the *B. licheniformis* enzyme. In the best conserved domain, the

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(α/β) barrel, most secondary structures were conserved, with the exception of helices α_3 and α_4 which were significantly shorter in the *Pyrococcus* enzyme (Fig. 2).

Another striking difference in the (α/β) barrel was the presence of two nine-residues deletions in loops $B_7 \rightarrow \alpha_7$ and $B_8 \rightarrow \alpha_8$ of the *Pyrococcus* enzyme (Fig. 2). The four active site consensus regions characteristic of the α -amylase family were present in the *P. furiosus* α -amylase (Fig. 2). From the three *B. licheniformis* α -amylase residues (Asn104, Asp200, and His235) involved in Ca_2 binding (28), only Asn104 was conserved in the *Pyrococcus* enzyme (Fig. 2). Interestingly, the two strictly conserved *B. licheniformis* α -amylase residues involved in chloride binding (Arg229 and Asn326) are present in the *Pyrococcus* enzyme (Fig. 2).

The amino acid compositions of the two enzymes (not shown) differed in two ways. (i) The *P. furiosus* enzyme was more negatively charged (net charge of -21) than the *B. licheniformis* enzyme (net charge of -8). This difference is mainly due to a lower number of Lys plus Arg residues in the *P. furiosus* enzyme (35 compared to 54 in the *B. licheniformis* enzyme), and is probably responsible for the two-units difference between the enzymes's isoelectric points (pIs of 4.78 and 6.83 for *P. furiosus* and *B. licheniformis* enzymes, respectively). (ii) The *P. furiosus* enzyme contained 5% more aromatic residues (18.5% against 13.7%) than the *B. licheniformis* enzyme.

P. furiosus extracellular α -amylase was also compared to the two other *P. furiosus* amylolytic enzymes sequenced so far, the *P. furiosus* intracellular α -amylase (26) and amylopullulanase (15). No significant similarity was found with either of these enzymes.

Purification of the *P. furiosus* extracellular α -amylase

The *P. furiosus* extracellular α -amylase was very thermostable. It did not lose any activity when the E.

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coli cell homogenate was treated at 100°C for 20 minutes. However, more than 50% of its activity was lost after centrifugation due to coprecipitation of the enzyme with cell debris and other denatured proteins. The precipitated enzyme remained active and was detected after resuspending the precipitate. Triton X-100 did not significantly prevent the protein from coprecipitating. To reduce coprecipitation, the cell homogenate was heated to 80°C for 15 minutes.

The α -amylase was so hydrophobic that it was directly absorbed onto the Phenyl-Sepharose column in the absence of any salt. The hydrophobic interaction was weakened by raising the buffer pH. The enzyme was totally eluted by 6 M urea at pH 9.4. The purified α -amylase displayed one protein band on native polyacrylamide gel (Fig. 3A) and had a specific activity of 3900 U/mg at 98°C.

Properties of the P. furiosus extracellular α -amylase vs TAKA-THERM

The approximate molecular weight of the recombinant *P. furiosus* extracellular α -amylase was 100,000 as estimated by gel filtration. This value was just twice the molecular weight calculated according to its deduced polypeptide sequence, indicating that the protein was a homodimer.

Experiments were initiated to test whether the α -amylase was active as a dimer or a monomer. Fig. 3 illustrates *P. furiosus* extracellular α -amylase behavior on 12% polyacrylamide native gel (A), SDS gel (B), and starch-containing SDS gel (C). Referring to Fig. 3, A: Protein sample was not denatured before loading, and B and C: Protein samples were treated in denaturing buffer at 60°C (lanes 1), 90°C (lane 2), 100°C (lane 3), or 110°C (lanes 4) for 10 minutes before loading. Gels A and B were stained by coomassie blue. Gel C was stained for α -amylase activity.

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Native gel showed one protein band (Fig. 3A). On SDS-PAGE, when denaturing temperatures were under 60°C the protein remained dimeric with an apparent molecular weight (MW) of 66,000. If denaturation was performed at 90°C or above, a 44,000 MW protein band appeared. This MW was lower than the 52,000 MW expected from the sequence. At 110°C, all the dimeric enzyme had dissociated into monomers of 44,000 MW along with protein degradation products. Both the dimer and monomer showed α -amylase activity (Fig. 3C). Due to the affinity of the dimeric enzyme for starch, the dimer migrated slower on starch-containing SDS gel than on SDS gel in the absence of starch. A low molecular weight protein band appeared at the bottom of SDS gels from protein degradation. Protein degradation in SDS buffers was also observed with other proteins (25).

TAKA-THERM was more sensitive to denaturation than the *P. furiosus*. During denaturation at or below 60°C, the protein retained its dimeric form with an apparent molecular weight of 122,000. Denaturing the enzyme at 90°C led to complete dissociation into monomers that migrated at 59,000 (Fig. 4A). TAKA-THERM only displayed α -amylase activity as a dimer (Fig. 4B).

Fig. 4 demonstrates TAKA-THERM α -amylase behavior on (A) 12% SDS-PAGE and (B) starch-containing 12% SDS-PAGE. Protein samples were treated in denaturing buffer at 60°C (lanes 1), or 90°C (lanes 2) for 10 minutes before loading. Gel A was stained by coomassie blue and gel B was stained for α -amylase activity.

Fig. 5 demonstrates the effect of temperature on the activities of *P. furiosus* extracellular α -amylase (●) and TAKA-THERM (■). TAKA-THERM was assayed in the presence of 0.5 mM Ca^{2+} . *P. furiosus* α -amylase displayed no activity at room temperature.

Referring to Fig. 5, α -amylase activity increased with temperature up to an optimum at 100°C. TAKA-THERM showed about 22% activity at room temperature and reached

its highest activity at 90°C. Both Arrhenius plots were linear (Fig. 5, inset). Activation energies were 70 kJ mole⁻¹ and 17 kJ mole⁻¹ for the *P. furiosus* α-amylase and TAKA-THERM, respectively, as calculated from the

5 Arrhenius equation: $\ln(k) = B - E_{\text{act}}/RT$ (where k = rate constant; B = constant; E_{act} = activation energy; R = molar gas constant [8.314 J mol⁻¹ K⁻¹]; and T = absolute temperature). Unlike TAKA-THERM's activity which

10 increased by approximately 10% in the presence of 0.5 mM Ca²⁺, the *Pyrococcus* enzyme did not require Ca²⁺ for activity.

The *P. furiosus* extracellular α-amylase had a lower optimal pH than TAKA-THERM (Fig. 6). Fig. 6 illustrates the influence of pH on *P. furiosus* α-amylase (●) and

15 TAKA-THERM (○) activities. *P. furiosus* α-amylase was assayed at 98°C without Ca²⁺, whereas TAKA-THERM was assayed at 90°C in the presence of 0.5 mM Ca²⁺.

The *Pyrococcus* enzyme showed 80% activity or more between pHs 4.5-7.0, with an optimal pH around 5.5-6.0.

20 TAKA-THERM was optimally active around pH 7.0-8.0.

Fig. 7 compares the thermostabilities of the recombinant *P. furiosus* extracellular α-amylase (■, ●) and TAKA-THERM (x, □) in the absence (■, ●, □) or presence (x) of 5 mM Ca²⁺ at 90°C (x, □, ■) or 98°C (●).

25 The *Pyrococcus* enzyme's inactivation time courses in the presence and absence of Ca²⁺ (not shown) were identical at the two temperatures tested (90 and 98°C). Ca²⁺, however, strongly stabilized TAKA-THERM. At 90°C, its half-life increased more than 20-fold in the presence

30 of 5 mM Ca²⁺. One hour incubation at 98°C completely inactivated TAKA-THERM, even in the presence of 5 mM Ca²⁺.

Substrate specificity and hydrolysis product analysis

The *P. furiosus* extracellular α-amylase hydrolyzed a wide variety of substrates such as soluble starch,

35 amylose, amylopectin, glycogen and oligosaccharides. The enzyme did not hydrolyze pullulan, cyclodextrins,

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sucrose, and maltose. α -Amylases can be classified into liquefying-type and saccharifying-type enzymes. Liquefying α -amylases have much wider commercial applications. Table 2 compares the hydrolysis products of *P. furiosus* and TAKA-THERM α -amylases. Like Taka-therm, the *P. furiosus* extracellular α -amylase was a liquefying enzyme. The main products of polysaccharide hydrolysis were G₂-G₇. A low amount of glucose was formed after long hydrolysis periods. The *P. furiosus* extracellular α -amylase hydrolyzed long-chain oligosaccharides faster than shorter chain oligosaccharides, as interpreted from the quantitation of products formed after short versus long incubation times (Table 3).

15 α -amylase overexpression in *E. coli*

Since very little α -amylase was produced from plasmid pS4 (about 1 mg/liter culture), we developed a construct that expressed more enzyme. In plasmid pET213, *P. furiosus* α -amylase gene was cloned under the control of the T7 promoter, without the sequence encoding its signal peptide. A band corresponding to a 45 kDa protein was observed on SDS-PAGE in crude extracts of BL21(DE3)(pET213) cultures after IPTG induction (not shown). This band was absent in crude extracts of uninduced BL21(DE3)(pET213) cultures. 38,000 U α -amylase activity at 98°C were obtained per liter of IPTG-induced BL21(DE3)(pET213), corresponding to the expression of 10 mg/liter α -amylase.

3. Discussion

With the cloning and characterization of the *P. furiosus* extracellular α -amylase described herein, three *P. furiosus* amylolytic enzymes--intracellular α -amylase (25; 26), extracellular α -amylase, and amylopullulanase (15)--have now been characterized. The new α -amylase appeared extracellular and belonged to the main family of

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α -amylases, as opposed to the α -amylase characterized by Laderman, et al. (25; 26) which was intracellular and did not show any similarity to the α -amylase family. The new enzyme did not display any pullulanase activity, and its
5 sequence was not related to *P. furiosus* amylopullulanase.

Koch, et al. (23) described an extracellular α -amylase activity present in the supernatant of *P. furiosus* cultures. The activity they described corresponded to two starch-degrading protein bands of 96
10 and 136 kDa on native polyacrylamide gel. It is not clear from their work if these two bands correspond to one or two separate enzymes and if the 96 kDa band is similar to the enzyme described here. It is unlikely that these bands are the *P. furiosus* amylopullulanase (8;
15 15), since no pullulanase activity was detected in Koch, et al.'s enzyme preparation (23).

Another member of the order *Thermococcales*, optimally growing at 80°C, *Thermococcus profundus* produced two extracellular amylases, amylases S and L
20 (11). With a molecular mass of 42 kDa on SDS-PAGE, amylase S is an α -amylase optimally active at pH 5.5-6.0 and 80°C, and does not require Ca^{2+} for its activity. While no sequence is available for amylase S, its catalytic properties suggest that this enzyme is the
25 counterpart in *T. profundus* of *P. furiosus* extracellular α -amylase. Amylase L, a bigger size enzyme, could correspond to the *P. furiosus* 136 kDa amylolytic enzyme detected by Koch, et al. (23).

We propose that the *P. furiosus* extracellular α -
30 amylase and amylopullulanase are involved in starch degradation. A putative integral membrane protein encoded by an ORF located upstream of the amylopullulanase gene (15) might participate in transporting the starch hydrolysis products inside the
35 cells where an α -glucosidase hydrolyses them to glucose (12). Because starch is typically an extracellular compound, the function of *P. furiosus* intracellular

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α -amylase is not clear. This enzyme can degrade starch down to glucose and maltose plus a mixture of oligosaccharides, most of them G_4 , G_5 , and G_6 . It is also able to synthesize G_4 and G_6 from maltose, and G_4 , G_5 , and G_6 from maltotriose (25). Its function might not be in starch catabolism. Several other starch-degrading hyperthermophilic eubacteria and archaea also contain two or more amylases. So far, though, all of them are extracellular (11; 18; 21; 23) or exposed on the cell surface (37).

The new *P. furiosus* α -amylase gene was preceded by a typical archaeal "TATA" box and a ribosome binding site. Generally, genes from hyperthermophilic archaea are not directly expressed in *E. coli*. Among all the genes reported from hyperthermophilic archaea, only *P. woesei* (35) and *P. furiosus* (15) amylopullulanase genes, *P. furiosus* β -glucosidase (45), and β -mannosidase (4) genes were expressed in *E. coli* from promoters present in their upstream non-coding sequences. Sequences reminiscent of -35 and -10 *E. coli* promoter sequences could be identified in front of most of these genes (see 15). One of the two sequences reminiscent of *E. coli* promoters and located upstream of the *P. furiosus* α -amylase gene probably allows the α -amylase to be expressed in *E. coli*. This observation supports our earlier hypothesis (15) that direct expression of archaeal genes in *E. coli* requires a sequence reminiscent of an *E. coli* promoter.

The *P. furiosus* extracellular α -amylase is the first archaeal amylolytic enzyme described belonging to the α -amylase family. The characterization of extracellular α -amylases with similar properties from other hyperthermophilic archaea (11; 24) suggests that these enzymes also belong to the α -amylase family and that this enzyme family is widespread among the three kingdoms. A second α -amylase family composed so far of only two enzymes, the *Dictyoglomus thermophilum* AmyA α -amylase and the *P. furiosus* intracellular α -amylase, spreads already

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in two kingdoms, eubacteria (*D. thermophilum*) and archaea (*P. furiosus*). It is not excluded that enzymes from this family be found in eukaryotes as well.

5 The *P. furiosus* α -amylase described here showed a high identity level to the *B. licheniformis* α -amylase. It contained the four consensus regions typical of the α -amylase family. With the exception of helices α_3 and α_4 , which were shorter, the secondary structures of the α/β barrel domain were well conserved, in particular the β strands and the β - α loops which are all located in the direct vicinity of the active site (Fig. 2). The least conserved domain was domain B which was 30% shorter than in *B. licheniformis* enzyme, and showed low sequence conservation (Fig. 2).

15 Since the *P. furiosus* extracellular α -amylase was significantly more thermostable than the commercial TAKA-THERM enzyme from *B. licheniformis*, the two enzymes sequences were compared to look for potential stabilizing elements in the *Pyrococcus* enzyme. (i) The *Pyrococcus* enzyme was significantly more negatively charged (by 13 charge units) than the *B. licheniformis* enzyme. It is not clear how this difference can affect the enzyme stability. It might involve additional surface interactions with extracellular salts. This charge difference might affect the enzymes's optimum pH for activity and stability more than their thermostability, though. There is indeed a two-pH-units difference between the two enzymes's pIs and also almost a two-pH-units difference between their optimum pHs for activity (pH 5.5 and 7.5 for *P. furiosus* and *B. licheniformis* enzymes, respectively).

(ii) The *P. furiosus* enzyme contains 5% more aromatic residues (18.5% against 13.7%) than the *B. licheniformis* enzyme. Aromatic residues have been shown to form networks of potentially stabilizing aromatic interactions in some thermostable enzymes (38). The three-dimensional structure of the *Pyrococcus* enzyme is

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probably required to study the extent to which the additional aromatic residues create new stabilizing interactions in this enzyme.

(iii) The *P. furiosus* enzyme is significantly shorter than its *B. licheniformis* counterpart. Increased enzyme compactness has often been suggested as a thermostabilizing factor (10). The variation in heat capacity (ΔC_p) associated with protein unfolding is considered to be essentially related to the change in solvent accessible surface area (in particular the exposure of hydrophobic residues to water) (see 29). Because smaller native proteins have a smaller solvent-accessible surface area, their ΔC_p of unfolding are reduced, their stability curves are broader, and their melting temperatures are higher (see 29). The areas affected by deletions in the *Pyrococcus* enzyme are mostly regions with little secondary structure or higher flexibility (i.e., the regions most susceptible to unfold first) such as loops β_7 - α_7 and β_8 - α_8 in the (α/β) barrel, or domain B, which is all together more susceptible to unfolding than the (α/β) barrel.

(iv) Two of the Ca^{2+} binding residues of *B. licheniformis* α -amylase (Asp200, and His235) are absent in the *Pyrococcus* enzyme. This absence is not surprising since the *Pyrococcus* enzyme does not require Ca^{2+} for either its activity or its stability. In the α -amylases whose three-dimensional structure has been solved, Ca^{2+} participates in stabilizing the interaction between the (α/β) barrel and domain B by creating an ionic bridge between the two domains (9; 28). Since domain B is poorly conserved and significantly shorter in *P. furiosus* α -amylase, and since Ca^{2+} is not required for the enzyme stability, a different type of interaction might exist between domains A and B in this enzyme, that does not involve a Ca^{2+} cation.

(v) Unlike the *B. licheniformis* enzyme which does not contain any cysteine residue, the *P. furiosus* α -

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amylase contains five cysteines, three in domain B and two in domain C. It is now well known that cysteine residues are among the residues most sensitive to degradation at high temperatures (44), and that they are usually rare in highly thermostable enzymes (48; 14). The presence of five cysteines in the *Pyrococcus* enzyme is, therefore, surprising. Chung, et al. (11) reported that an accessible cysteine may be involved in *T. profundus* α -amylase catalysis. A similar situation can happen with the *Pyrococcus* enzyme if, as we think, the *Thermococcus* and *Pyrococcus* extracellular α -amylases are related.

Denaturation of the *P. furiosus* α -amylase dimer required harsh denaturing conditions. It required 2% SDS plus 0.64 M mercaptoethanol and temperatures above 90°C to dissociate into monomers. Unfolding was not complete, though, since the enzyme remained at least partially active. The apparently low MW of 44,000 (as compared to a 52,000 MW predicted from the sequence) observed for the monomer in these conditions might reflect the incomplete unfolding of the enzyme. Such a behavior was not observed with the *B. licheniformis* enzyme which, once it was dissociated into monomers, had an apparent 59,000 MW on SDS-PAGE identical to the 58,500 MW predicted from its sequence, and was completely inactive (Fig. 4).

Denaturation of the *P. furiosus* α -amylase dimer into the monomeric form occurred at temperatures twenty degrees higher than required for the TAKA-THERM enzyme. Notably, the monomer of *P. furiosus* α -amylase remained active, whereas the TAKA-THERM monomer was completely inactive. The apparent molecular weight of the *P. furiosus* extracellular α -Amylase dimer on SDS-PAGE was only 1.5 times higher than its monomer, indicating that the protein still retained its globular structure and moved faster on SDS-PAGE than expected from its actual molecular weight. This behavior was also observed with other proteins from hyperthermophiles (15; 35).

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A *P. woesei* extracellular α -amylase has been purified and characterized by Koch, et al. (24). *P. furiosus* and *P. woesei* extracellular α -amylases are optimally active in the same conditions of pH and temperature and have similar resistance to thermal inactivation. Although the *P. woesei* enzyme was described as a 70,000 molecular weight enzyme--as indicated from migration on an SDS-PAGE--it could correspond to a dimeric enzyme showing an aberrant behavior in these electrophoresis conditions. Such a behavior was observed with the *P. furiosus* enzyme, when mild denaturation conditions were used. It migrated as a 66,000 molecular weight dimer, instead of a 52,000 monomer. *P. furiosus* and *P. woesei* extracellular α -Amylases seem to differ on two aspects. (i) The *P. woesei* enzyme shows almost six-times less specific activity than the *P. furiosus* enzyme (667 versus 3900 U mg⁻¹). (ii) Their amino acid compositions seem to be different. In particular, the *P. furiosus* enzyme contains half the threonine residues present in the *P. woesei* enzyme. *P. furiosus* and *P. woesei* amylopullulanases were shown to be significantly different in a few aspects (15). Although these two organisms are considered very close, they still contain quite different enzymes.

Table 2 summarizes the differences between extracellular α -Amylases from *P. furiosus* and *B. licheniformis* (TAKA-THERM). Starch liquefying requires using α -amylase at high temperatures (up to 110°C). TAKA-THERM has a wide application in industry today. *P. furiosus* extracellular α -amylase showed promising properties over TAKA-THERM: (1) The enzyme displayed higher optimal temperature and thermostability than TAKA-THERM. Therefore, starch liquefying can be performed at very high temperatures without a risk of losing activity; (2) The enzyme had a low optimal pH (pH 5.5 versus pH 7.5). Thus, starch liquefying and saccharifying can be

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operated under more similar pH conditions. (3) Unlike TAKA-THERM, Ca^{2+} was not needed for activity and thermostability. This could eliminate the ion-exchange step used to remove Ca^{2+} which is an inhibitor during high fructose syrup production via glucose isomerase. (4) The *P. furiosus* enzyme is about twice as active as TAKA-THERM.

Table 1: Hydrolysis products of different substrates by the recombinant *P. furiosus* and Taka-therm α -amylases

Substrate	Enzyme sources	Product (%)*							
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	G ₇	> G ₇
Soluble Starch	<i>P. furiosus</i>	2.0	27.5	18.2	12.1	16.4	16.0	11.4	5.0
	Taka-therm	13.5	31.9	18.2	11.5	16.5	6.1	2.4	1.1
Glycogen	<i>P. furiosus</i>	1.0	15.7	14.9	14.7	15.4	15.1	7.8	15.3
	Taka-therm	9.9	26.4	13.7	12.6	14.6	5.8	4.9	12.9
Amylose	<i>P. furiosus</i>	2.4	41.7	23.2	11.4	11.9	9.5	0.0	0.0
	Taka-therm	15.4	37.5	22.1	10.3	14.6	0.0	0.0	0.0

*Incubation period was 46 hours.

Table 2: *P. furiosus* α -amylase product specificity: comparison of the hydrolysis products of different oligosaccharides.

Substrate	Time Period (h)	Product/residual substrate (%)						
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	G ₇
Maltotetraose	0.83	0.0	2.3	13.0	84.8			
	46	0.2	25.2	24.7	49.5			
Maltopentaose	0.83	0.0	2.0	3.0	11.3	83.7		
	46	0.6	19.2	24.4	21.1	35.0		
Maltohexaose	0.83	0.1	1.5	1.8	2.0	14.2	80.4	
	46	1.6	18.3	18.2	14.5	19.2	28.1	
Maltoheptaose	0.83	1.2	4.2	0.9	1.7	9.1	15.6	67.4
	46	3.64	32.6	12.4	14.0	23.0	13.5	0.9

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Table 3. Comparison of the general biochemical properties of the *P. furiosus* extracellular and *B. licheniformis* Taka-therm α -amylases

	Properties	<i>P. furiosus</i>	TAKA-THERM
5	Molecular weight	100,000	122,000
	Specific activity (U/mg)	3,900	2,000
	Subunit activity	Yes	No
	Optimal pH	5.5-6.0	7.0-8.0
	Optimal temperature	100°C	90°C
10	Ca ²⁺ requirement	No	Yes
	Half-life at 98°C	13 h	< 1 hr
	End product	G ₂ -G ₇	G ₁ -G ₆

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REFERENCES

1. Adams, M.W.W., "Enzymes and proteins from organisms that grow near and above 100°C," Ann. Rev. Microbiol. 47:627-658, 1993.
2. Adams, M.W.W., F.B. Perler, and R.M. Kelly, "Extremozymes: expanding the limits of biocatalysis," Bio/Technology 13:662-668, 1995.
3. Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (eds.), "Current Protocols in Molecular Biology," Greene Publishing & Wiley-Interscience, New York, 1993.
4. Bauer M.W., E.J. Bylina, R.V. Swanson, and R.M. Kelly, "Comparison of a β -glucosidase and a β -mannosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*: purification, characterization, gene cloning and sequence analysis," J. Biol. Chem. 271:23749-23755, 1996.
5. Bernfeld, P., "Amylases α - and β -," Methods Enzymol. 1:149-158, 1955.
6. Boel, E.L., Brady, A.M., Brzozowski, Z., Derewenda, G.G., Dogson, V.J., Jensen, S.B., Petersen, H., Swift, L., Thim, and H.F. Woldike, "Calcium binding in α -amylases: An X-ray diffraction study at 2.1-Å resolution of two enzymes from *Aspergillus*," Biochemistry 29:6244-6249, 1990.
7. Brown, S.H., H.R. Costantino, and R.M. Kelly, "Characterization of amylolytic activities associated with the hyperthermophilic archaeobacterium *Pyrococcus furiosus*," Appl. Environ. Microbiol. 56:1985-1991, 1990.
8. Brown, S.H., and R.M. Kelly, "Characterization of amylolytic enzymes, having both α -1,4 and α -1,6 hydrolytic activity, from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*," Appl. Environ. Microbiol. 59:2614-2621, 1993.
9. Buisson, G., E. Duee, R. Haser, and F. Payan, "Three dimensional structure of porcine pancreatic α -amylase at 2.9 Å resolution. Role of calcium in structure and activity," EMBO J. 6:3909-3916, 1987.
10. Chan, M.K., S. Mukund, A. Kletzin, M.W.W. Adams, and D.C. Rees, "Structure of a hyperthermophilic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase," Science 267:1463-1469, 1995.

-31-

11. Chung, Y.C., T. Kobayashi, H. Kanai, T. Akiba, and T. Kudo, "Purification and Properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432," Appl. Environ. Microbiol. 61:1502-1506, 1995.
12. Costantino, H.R., S.H. Brown, and R.M. Kelly, "Purification and characterization of an α -glucosidase from a hyperthermophilic archaeobacterium, *Pyrococcus furiosus*, exhibiting a temperature optimum of 105 to 115°C," J. Bacteriol. 172:3654-3660, 1990.
13. Devereux, J., P. Haeberli, and O. Smithies, "A comprehensive set of sequence analysis programs for the VAX." Nucleic Acids Res. 12:387-395, 1984.
14. DiRuggiero, J., F.T. Robb, R. Jagus, H.H. Klump, K.M. Borgest, M. Kessel, X. Mai, and M.W.W. Adams, "Characterization, cloning, and *in vitro* expression of the extremely thermostable glutamate dehydrogenase from the hyperthermophilic archaeon, ES4," J. Biol. Chem. 268:17767-17774, 1993.
15. Dong, G., C. Vieille, and J.G. Zeikus, "Cloning, sequencing and expression of the gene encoding the *Pyrococcus furiosus* amylopullulanase and enzyme characterization," Submitted to Appl. Environ. Microbiol., 1997.
16. Fiala, G., and K.O. Stetter, "*Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaeobacteria growing optimally at 100°C," Arch. Microbiol. 145:56-61, 1986.
17. Fogarty, W.M., and C.T. Kelly, "Recent advances in microbial amylases," In Fogarty W.M., and C.T. Kelly (eds.), *Microbial Enzymes and Biotechnology*, 2nd edition, pp 71-121, Elsevier Applied Science, New York, 1990.
18. Fukusumi, S., A. Kamizono, S. Horinouchi, and T. Beppu, "Cloning and nucleotide sequence of a heat-stable amylase gene from an anaerobic thermophile, *Dictyoglomus thermophilum*," Eur. J. Biochem. 174:15-21, 1988.
19. Hain, J., W.-D. Reiter, U. Hudepohl, and W. Zillig, "Elements of an archaeal promoter defined by mutational analysis," Nucleic Acids Res. 20:5423-5428, 1992.
20. Henikoff, S., "Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing," Gene 28:351-359, 1984.

-32-

21. Horinouchi, S., S. Fukusumi, T. Ohshima, and T. Beppu, "Cloning and expression in *Escherichia coli* of two additional amylase genes of a strictly anaerobic thermophile, *Dictyoglomus thermophilum*, and their nucleotide sequences with extremely low guanine-plus-cytosine contents," Eur. J. Biochem. 176:243-253, 1988.
22. Jespersen, H.M., E.A. MacGregor, B. HenAssat, M.R. Sieks, and B. Svensson, "Starch- and glycogen-debranching and branching enzymes: prediction of structural features of the catalytic (β/α)₈-barrel domain and evolutionary relationship of other amylolytic enzymes," J. Prot. Chem. 12:791-805, 1993.
23. Koch, R., P. Zabłowski, A. Spreinat, and G. Antranikian, "Extremely thermostable amylolytic enzyme from archaeobacterium *Pyrococcus furiosus*," FEMS Microbiol. Letters 71:21-26, 1990.
24. Koch, R., A. Sprehat, K. Lemke, and G. Antranikian, "Purification and properties of a hyperthermoactive α -Amylase from archaeobacterium *Pyrococcus woesei*," Arch. Microbiol. 155:572-578, 1991.
25. Laderman, K.A., B.R. Davis, H.C. Krutzsch, M.S. Lewis, Y.V. Griko, P.L. Privalov, and C.B. Anfinsen, "The purification and characterization of an extremely thermostable α -Amylase from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*," J. Biol. Chem. 268:24394-24401, 1993a.
26. Laderman, K.A., K. Asada, T. Uemori, H. Mukai, Y. Taguchi, I. Kato, and C.B. Anfinsen, α -Amylase from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*, cloning and sequencing of the gene and expression in *Escherichia coli*," J. Biol. Chem. 268:24402-24407, 1993b.
27. Laemmli, U.K., "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," Nature 227:680-685, 1970.
28. Machius, M., G. Wiegand, and R. Huber, "Crystal structure of calcium-depleted *Bacillus licheniformis* α -Amylase at 2.2 Å resolution," J. Mol. Biol. 246:545-559, 1995.
29. McCrary, B.S., S.P. Edmondson, and J.W. Shriver, "Hyperthermophile protein folding thermodynamics: differential scanning calorimetry and chemical denaturation of Sac7d," J. Mol. Biol. 264:784-805, 1996.

30. Perrin, D.D., and B. Dempsey (eds.), "Buffers for pH and Metal ion control," Chapman and Hall Ltd, London, 1979.
31. Qian, M., R. Haser., G. Buisson, E. Duée, and F. Payan, "The active center of a mammalian α -amylase. Structure of the complex of a pancreatic α -amylase with a carbohydrate inhibitor refined to 2.2 Å," Biochem. 33:6284-6294, 1994.
32. Qian, M., R. Haser, and F. Payan, "Structure and molecular model refinement of pig pancreatic α -amylase at 2.1 Å resolution," J. Mol. Biol. 231:785-799, 1993.
33. Reiter, W.-D., P. Palm, and W. Zillig, "Transcription termination in the archeabacterium *Sulfolobus*: signal structures and linkage to transcription initiation," Nucl. Acids Res. 16:2445-2459, 1988.
34. Robinson, K.A., and H.J. Schreier, "Isolation, sequence and characterization of the maltose-regulated *mlrA* gene from hyperthermophilic archaeum *Pyrococcus furiosus*," Gene 151:173-176, 1994.
35. Rüdiger, A., P.L. Jorgensen, and G. Antranikian, "Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*," Appl. Environ. Microbiol. 61:567-575, 1995.
36. Sanger, F., S. Nicklen, and A.R. Coulson, "DNA sequencing with chain terminating inhibitors," Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977.
37. Schumann, J., A. Wrba, R. Jaenicke, and K.O. Stetter, "Topographical and enzymatic characterization of amylases from the extremely thermophilic eubacterium *Thermotoga maritima*," FEBS Lett. 282:122-126, 1991.
38. Teplyakov, A.V., I.P. Kuranova, E.H. Harutyunyan, B.K. Vainshtein, C. Frömmel, W.E. Höhne, and K.S. Wilson, "Crystal structure of thermitase at 1.4 Å resolution," J. Mol. Biol. 214:261-279, 1990.
39. Tiboni, O., P. Cammarano, and A.M. Sanangelantoni, "Cloning and sequencing of the gene encoding glutamine synthetase I from the archaeum *Pyrococcus woesei*: anomalous phylogenies inferred from analysis of archaeal and bacterial glutamine synthetase I sequences," J. Bacteriol. 175:2961-2969, 1993.

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40. Tomazic, S.J., and A.M. Klibanov, "Mechanisms of irreversible thermal inactivation of *Bacillus* α -amylases," J. Biol. Chem. 263:3086-3091, 1988a.
41. Tomazic, S.J., and A.M. Klibanov, "Why is one *Bacillus* α -amylase more resistant against irreversible thermoinactivation than another"? J. Biol. Chem. 263:3092-3096, 1988b.
42. Vieille, C., D.S. Burdette, and J.G. Zeikus, "Thermozymes," Biotechnol. Annual Rev. 2:1-83, 1996.
43. Violet, M., and J.-C. Meunier, "Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α -amylase," Biochem. J. 263:665-670, 1989.
44. Volkin, D.B., and C.R. Middaugh, "The effect of temperature on protein structure," In Ahern, T.J., and M.C. Manning (eds.), *Stability of Protein Pharmaceuticals, Part A: Chemical and Physical Pathways of Protein Degradation*, Plenum Press, New York, 1992.
45. Voorhorst, W.G.B., R.I.L. Eggen, E.J. Luesink, and W.M. de Vos, "Characterization of the *celB* gene coding for β -glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its expression and site-directed mutation in *Escherichia coli*," J. Bacteriol. 177:7105-7111, 1995.
46. Voorhorst, W.G.B., R.I.L. Eggen, A.C.M. Geerling, C. Platteeuw, R.J. Siezen, and W.M. de Vos, "Isolation and characterization of the hyperthermostable serine protease, pyrolysin, and its gene from the hyperthermophilic archaeon *Pyrococcus furiosus*," J. Biol. Chem. 271:20426-20431, 1996.
47. Watson, M.E.E., "Compilation of published signal sequences," Nucleic Acids Res. 12:5145-5264, 1984.
48. Zwickl, P., S. Fabry, C. Bogedain, A. Haas, and R. Hensel, "Glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic archaeobacterium *Pyrococcus woesei*: characterization of the enzyme, cloning and sequencing of the gene, and expression in *Escherichia coli*," J. Bacteriol. 172:4329-4338, 1990.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Michigan Biotechnology Institute
 (B) STREET: 3900 Collins Road
 (C) CITY: Lansing
 (D) STATE: Michigan
 (E) COUNTRY: United States of America
 (F) POSTAL CODE: 48909
 (G) TELEPHONE: (517) 337-3181
 (H) TELEFAX: (517) 337-2122

(ii) TITLE OF INVENTION: HYPERTHERMOSTABLE α -AMYLASE

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Quarles & Brady
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 (C) CITY: Milwaukee
 (D) STATE: Wisconsin
 (E) COUNTRY: U.S.A.
 (F) ZIP: 53202-4497

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
 (B) FILING DATE:
 (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Baker, Jean C.
 (B) REGISTRATION NUMBER: 35,433
 (C) REFERENCE/DOCKET NUMBER: 660336.90641

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (414) 277-5709
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 460 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Val Asn Ile Lys Lys Leu Thr Pro Leu Leu Thr Leu Leu Leu Phe Phe
1           5           10           15

Ile Val Leu Ala Ser Pro Val Ser Ala Ala Lys Tyr Leu Glu Leu Glu
20          25          30

Glu Gly Gly Val Ile Met Gln Ala Phe Tyr Trp Asp Val Pro Gly Gly
35          40          45

Gly Ile Trp Trp Asp His Ile Arg Ser Lys Ile Pro Glu Trp Tyr Glu
50          55          60

Ala Gly Ile Ser Ala Ile Trp Leu Pro Pro Pro Ser Lys Gly Met Ser
65          70          75          80

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Gly	Gly	Tyr	Ser	Met 85	Gly	Tyr	Asp	Pro	Tyr 90	Asp	Tyr	Phe	Asp	Leu 95	Gly
Glu	Tyr	Tyr	Gln 100	Lys	Gly	Thr	Val	Glu 105	Thr	Arg	Phe	Gly	Ser 110	Lys	Glu
Glu	Leu	Val 115	Arg	Leu	Ile	Gln	Thr 120	Ala	His	Ala	Tyr	Gly 125	Ile	Lys	Val
Ile	Ala 130	Asp	Val	Val	Ile	Asn 135	His	Arg	Ala	Gly	Gly 140	Asp	Leu	Glu	Trp
Asn 145	Pro	Phe	Val	Gly	Asp 150	Tyr	Thr	Trp	Thr	Asp 155	Phe	Ser	Lys	Val	Ala 160
Ser	Gly	Lys	Tyr	Thr 165	Ala	Asn	Tyr	Leu	Asp 170	Phe	His	Pro	Asn	Glu 175	Leu
His	Cys	Cys	Asp 180	Glu	Gly	Thr	Phe	Gly 185	Gly	Phe	Pro	Asp	Ile 190	Cys	His
His	Lys	Glu 195	Trp	Asp	Gln	Tyr	Trp 200	Leu	Trp	Lys	Ser	Asn 205	Glu	Ser	Tyr
Ala	Ala 210	Tyr	Leu	Arg	Ser	Ile 215	Gly	Phe	Asp	Gly	Trp 220	Arg	Phe	Asp	Tyr
Val 225	Lys	Gly	Tyr	Gly	Ala 230	Trp	Val	Val	Arg	Asp 235	Trp	Leu	Asn	Trp	Trp 240
Gly	Gly	Trp	Ala 245	Val	Gly	Glu	Tyr	Trp	Asp 250	Thr	Asn	Val	Asp	Ala 255	Leu
Leu	Ser	Trp	Ala 260	Tyr	Glu	Ser	Gly	Ala 265	Lys	Val	Phe	Asp	Phe 270	Pro	Leu
Tyr	Tyr	Lys 275	Met	Asp	Glu	Ala	Phe 280	Asp	Asn	Asn	Asn	Ile 285	Pro	Ala	Leu
Val	Tyr 290	Ala	Leu	Gln	Asn	Gly 295	Gln	Thr	Val	Val	Ser 300	Arg	Asp	Pro	Phe
Lys 305	Ala	Val	Thr	Phe	Val 310	Ala	Asn	His	Asp	Thr 315	Asp	Ile	Ile	Trp	Asn 320
Lys	Thr	Pro	Ala	Tyr 325	Ala	Phe	Ile	Leu	Thr 330	Tyr	Glu	Gly	Gln	Pro 335	Val
Ile	Phe	Tyr	Arg 340	Asp	Phe	Glu	Glu	Trp 345	Leu	Asn	Lys	Asp	Lys 350	Leu	Ile
Asn	Leu	Ile 355	Trp	Ile	His	Asp	His 360	Leu	Ala	Gly	Gly	Ser 365	Thr	Thr	Ile
Val	Tyr 370	Tyr	Asp	Asn	Asp	Glu 375	Leu	Ile	Phe	Val	Arg 380	Asn	Gly	Asp	Ser
Arg 385	Arg	Pro	Gly	Leu	Ile 390	Thr	Tyr	Ile	Asn	Leu 395	Ser	Pro	Asn	Trp	Val 400
Gly	Arg	Trp	Val	Tyr 405	Val	Pro	Lys	Phe	Ala 410	Gly	Ala	Cys	Ile	His 415	Glu
Tyr	Thr	Gly	Asn 420	Leu	Gly	Gly	Trp	Val 425	Asp	Lys	Arg	Val	Asp 430	Ser	Ser
Gly	Trp	Val 435	Tyr	Leu	Glu	Ala	Pro 440	Pro	His	Asp	Pro	Ala 445	Asn	Gly	Tyr
Tyr	Gly 450	Tyr	Ser	Val	Trp	Ser 455	Tyr	Cys	Gly	Val	Gly 460				

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(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1542 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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TTTATTAGAT TTGACGTGC GTTGATGAAC ATTTATGTTT ACATGATCAT AACAGAAAAT      60
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AACATAAAGA AATTAACACC CCTCCTAACT CTATTACTGT TTTTATAGT ACTAGCAAGT      180
CCAGTAAGTG CAGCAAAATA CTTGGAGCTT GAAGAGGGAG GAGTTATAAT GCAAGCATTC      240
TATTGGGATG TTCCAGGGGG AGGAATTTGG TGGGATCATA TAAGATCGAA GATTCCTGAA      300
TGGTATGAAG CTGGAATCTC TGCAATATGG CTACCTCCAC CAAGCAAGGG GATGAGTGGA      360
GGATATTCAA TGGGCTACGA TCCCTATGAT TACTTTGATC TCGGCGAGTA CTACCAGAAG      420
GGAACGTAG AGACGCGTTT TGGATCAAAA GAAGAACTAG TGAGATTGAT ACAAAGTCC      480
CATGCCTATG GAATAAAGGT AATCGCCGAT GTAGTTATAA ACCACAGGGC TGGTGGTGAC      540
CTAGAATGGA ACCCCTTCGT TGGAGATTAC ACATGGACAG ACTTTTCTAA AGTTCCTCA      600
GGGAAATATA CAGCTAACTA TCTGGACTTC CATCAAACG AGCTTCATTG TTGTGACGAA      660
GGAACCTTTG GAGGATTTCC AGATATATGT CATCACAAG AGTGGGATCA GTACTGGCTA      720
TGGAAGAGCA ATGAGAGTTA TGCTGCTTAT TTAAGAAGCA TAGGATTGA TGGTTGGAGA      780
TTTGACTATG TTAAGGGCTA TGGAGCTTGG GTTGTCAGAG ACTGGCTTAA TTGGTGGGGA      840
GGTTGGGCAG TTGGAGAGTA CTGGGACACA AATGTAGATG CACTACTAAG CTGGGCATAT      900
GAGAGTGGTG CAAAGGTCTT TGAAGTCCCG CTCTACTATA AAATGGATGA AGCATTTGAC      960
AATAACAACA TTCCAGCATT AGTCTATGCC CTACAAAACG GACAACTGT AGTTTCGAGA     1020
GATCCATTTA AGGCAGTAAC TTTCGTTGCC AATCATGACA CAGATATAAT ATGGAACAAG     1080
TATCCAGCAT ATGCGTTCAT ATTGACATAT GAGGGACAGC CAGTAATATT CTACAGGGAC     1140
TTTGAGGAAT GGCTGAACAA GGATAAGCTA ATTAACCTCA TTTGGATCCA TGATCATTTG     1200
GCAGGAGGAA GCACAACAAT TGTCTACTAC GACAACGATG AGCTCATATT TGTGAGAAAT     1260
GGAGATTCTA GAAGGCCTGG GCTTATAACT TACATTAACT TGAGCCCTAA CTGGGTTGGT     1320
AGGTGGGTAT ACGTTCCAAA GTTTGCAGGG GCTTGTTATC ATGAATACAC TGGAAACCTA     1380
GGAGGATGGG TAGATAAAG AGTAGATAGT AGCGGATGGG TATACCTAGA GGCACCACCT     1440
CACGATCCAG CTAACGGCTA CTATGGGTAC TCCGTATGGA GTTATTGTGG TGTGGGTGA     1500
CTTTTCTTT TTTCTTTTTA ACAATGGGAG AAGTGCAAAT AC                        1542

```

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 510 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Lys	Gln	Gln	Lys	Arg	Leu	Tyr	Ala	Arg	Leu	Leu	Thr	Leu	Leu	Phe	1	5	10	15
Ala	Leu	Ile	Phe	Leu	Leu	Pro	His	Ser	Ala	Ala	Ala	Ala	Ala	Asn	Leu	20	25	30	
Asn	Gly	Thr	Leu	Met	Gln	Tyr	Phe	Glu	Trp	Tyr	Met	Pro	Asn	Asp	Gly	35	40	45	
Gln	His	Trp	Lys	Arg	Leu	Gln	Asn	Asp	Ser	Ala	Tyr	Leu	Ala	Glu	His	50	55	60	
Gly	Ile	Thr	Ala	Val	Trp	Ile	Pro	Pro	Ala	Tyr	Lys	Gly	Thr	Ser	Gln	65	70	75	80
Ala	Asp	Val	Gly	Tyr	Gly	Ala	Tyr	Asp	Leu	Tyr	Asp	Leu	Gly	Glu	Phe	85	90	95	
His	Gln	Lys	Gly	Thr	Val	Arg	Thr	Lys	Tyr	Gly	Thr	Lys	Gly	Glu	Leu	100	105	110	
Ser	Ala	Ile	Lys	Ser	Leu	His	Ser	Arg	Asp	Ile	Asn	Val	Tyr	Gly	Asp	115	120	125	
Val	Val	Ile	Asn	His	Lys	Gly	Gly	Ala	Asp	Ala	Thr	Glu	Asp	Val	Thr	130	135	140	
Ala	Val	Glu	Val	Asp	Pro	Ala	Asp	Arg	Asn	Arg	Val	Ile	Ser	Gly	Glu	145	150	155	160
His	Arg	Ile	Lys	Ala	Trp	Thr	His	Phe	His	Phe	Pro	Gly	Arg	Gly	Ser	165	170	175	
Thr	Tyr	Ser	Asp	Phe	Lys	Trp	His	Trp	Tyr	His	Phe	Asp	Gly	Thr	Asp	180	185	190	
Trp	Asp	Glu	Ser	Arg	Lys	Leu	Asn	Arg	Ile	Tyr	Lys	Phe	Gln	Gly	Lys	195	200	205	
Ala	Trp	Asp	Trp	Glu	Val	Ser	Asn	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	210	215	220	
Met	Tyr	Ala	Asp	Ile	Asp	Tyr	Asp	His	Pro	Asp	Val	Ala	Ala	Glu	Ile	225	230	235	240
Lys	Arg	Trp	Gly	Thr	Trp	Tyr	Ala	Asn	Glu	Leu	Gln	Leu	Asp	Gly	Phe	245	250	255	
Arg	Leu	Asp	Ala	Val	Lys	His	Ile	Lys	Phe	Ser	Phe	Leu	Arg	Asp	Trp	260	265	270	
Val	Asn	His	Val	Arg	Glu	Lys	Thr	Gly	Lys	Glu	Met	Phe	Thr	Val	Ala	275	280	285	
Glu	Tyr	Trp	Gln	Asn	Asp	Leu	Gly	Ala	Leu	Glu	Asn	Tyr	Leu	Asn	Lys	290	295	300	
Thr	Asn	Phe	Asn	His	Ser	Val	Phe	Asp	Val	Pro	Leu	His	Tyr	Gln	Phe	305	310	315	320
His	Ala	Ala	Ser	Thr	Gln	Gly	Gly	Gly	Tyr	Asp	Met	Arg	Lys	Leu	Leu	325	330	335	
Asn	Ser	Thr	Val	Val	Ser	Lys	His	Pro	Leu	Lys	Ala	Val	Thr	Phe	Val	340	345	350	
Asp	Asn	His	Asp	Thr	Gln	Pro	Gly	Gln	Ser	Leu	Glu	Ser	Thr	Val	Gln	355	360	365	

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Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu Ser
 370 375 380
 Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly Asp
 385 390 395 400
 Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile Leu
 405 410 415
 Lys Ala Arg Lys Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe Asp His
 420 425 430
 His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val Ala Asn
 435 440 445
 Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala Lys Arg
 450 455 460
 Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp Ile Thr
 465 470 475 480
 Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp Gly Glu
 485 490 495
 Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
 500 505 510

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAAATGTCAC GTTGTATGG

19

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAGAGTGGTG CAAAGGTC

18

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGGTGGTGA CCTAGAATG

19

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(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TATCTGTGTC ATGATTGGC

19

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGGTGATCA CATGAACATA AAGAAATIAA CACC

33

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGTGTTAATT TCTTTATGTT CATGTGATCA CCT

33

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCTAGCTTG GAGCTTGAAG AGGGAG

26

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTCGAGACC ACAATAACTC CATACGGAG

29

- 41 -

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTGGG

5

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTCGAG

6

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CLAIMS

We claim:

1. A preparation of *Pyrococcus furiosus* extracellular α -amylase.
2. The preparation of claim 1, wherein the α -amylase has an optimal temperature of 100°C.
3. The preparation of claim 1, wherein the α -amylase has a pH optimum of 5.5-6.0.
4. The preparation of claim 1, wherein the α -amylase has a half-life at 98°C of between 12-14 hours.
5. The enzyme of claim 1, wherein the amino acid sequence of the enzyme comprises SEQ ID NO:1.
6. A preparation of α -amylase enzyme, wherein the enzyme has an optimal temperature of 100°C.
7. The enzyme of claim 6 wherein the enzyme has a pH optimum of 5.5-6.0.
8. The enzyme of claim 6, wherein the enzyme has a half-life at 98°C of 12-14 hours.
9. The enzyme of claim 6, wherein the enzyme has a molecular weight of 95,000-105,000, as measured by gel electrophoresis.
10. A method of hydrolyzing starch comprising the step of mixing starch and the α -Amylase of claim 1 under conditions suitable for enzyme activity, wherein the starch is degraded into G₂-G, oligosaccharides.

11. A method of hydrolyzing starch comprising the step of mixing starch and the α -Amylase of claim 6 under conditions suitable for enzyme activity, wherein the starch is degraded into G₂-G, oligosaccharides.

12. A method of creating an α -amylase preparation comprising

(a) obtaining a nucleotide sequence encoding an α -amylase, wherein the encoded enzyme has an optimal
5 temperature of 100°C, and

(b) placing the gene sequence into an expression vector, so that the sequence is expressed as a mature protein.

13. The method of claim 12 wherein the gene sequence is obtained from *Pyrococcus furiosus*.

14. The method of claim 13 wherein the gene sequence comprises residues 118 through 1497 of SEQ ID NO:2.

15. An isolated DNA sequence encoding an extracellular α -amylase, wherein the α -amylase has an optimal temperature of 100°C.

16. The sequence of claim 15 wherein the sequence is obtained from *Pyrococcus furiosus*.

17. The sequence of claim 16, wherein the sequence comprises SEQ ID NO:2.

18. The sequence of claim 16, wherein the sequence comprises residues 118 through 1497 of SEQ ID NO:2.

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1
 TTTATTAGATTTTGGACGTGCGTTGATGAACATTTATGTTCACATGATCATAACAGAAAAAT
 -35
 Box A RBS
TTATATGTATCATCACCAGTGATACATTATGAGACTTTGGTGTATGGAGGTGATCACGTG
 -10/-35 -10 V
 121
 AACATAAAGAAATTAACACCCCTCCTAACTCTATTACTGTTTTTTATAGTACTAGCAAGT
 N I K K L T P L L T L L L F F I V L A S
 181
 CCAGTAAGTGCAGCAAAATACTTGGAGCTTGAAGAGGGAGGAGTTATAATGCAAGCATTG
 P V S A A K Y L E L E E G G V I M Q A F
 241
 TATTGGGATGTTCCAGGGGAGGAATTTGGTGGGATCATATAAGATCGAAGATTCTCTGAA
 Y W D V P G G G I W W D H I R S K I P E
 301
 TGGTATGAAGCTGGAATCTCTGCAATATGGCTACCTCCACCAAGCAAGGGGATGAGTGGGA
 W Y E A G I S A I W L P P P S K G M S G
 361
 GGATATTCAATGGGCTACGATCCCTATGATTACTTTGATCTCGGCGAGTACTACCAGAAG
 G Y S M G Y D P Y D Y F D L G E Y Y Q K
 421
 GGAAGCTGTAGAGACGCGTTTTGGATCAAAAGAAGAACTAGTGAGATTGATACAACTGCC
 G T V E T R F G S K E E L V R L I Q T A
 481
 CATGCCTATGGAATAAAGGTAATCGCCGATGTAGTTATAAACCACAGGGCTGGTGGTGAC
 H A Y G I K V I A D V V I N H R A G G D
 541
 CTAGAATGGAACCCCTTCGTTGGAGATTACACATGGACAGACTTTTCTAAAGTTGCCTCA
 L E W N P F V G D Y T W T D F S K V A S
 601
 GGGAAATATACAGCTAACTATCTGGACTTCCATCCAAACGAGCTTCATTGTTGTGACGAA
 G K Y T A N Y L D F H P N E L H C C D E
 661
 GGAACCTTTGGAGGATTTCCAGATATATGTCATCACAAAGAGTGGGATCAGTACTGGCTA
 G T F G G F P D I C H H K E W D Q Y W L
 721
 TGGAAGAGCAATGAGAGTTATGCTGCTTATTTAAGAAGCATAGGATTTGATGGTTGGAGA
 W K S N E S Y A A Y L R S I G F D G W R
 781
 TTTGACTATGTAAAGGGCTATGGAGCTTGGGTTGTGAGAGACTGGCTTAATTGGTGGGGA
F D Y V K G Y G A W V V R D W L N W W G
 841 II
 GGTGGGCGAGTTGGAGAGTACTGGGACACAAATGTAGATGCACTACTAAGCTGGGCATAT
 G W A V G E Y W D T N V D A L L S W A Y
 901 III
 GAGAGTGGTGCAAAGGTCTTTGACTTCCCGCTCTACTATAAAATGGATGAAGCATTGAC
 E S G A K V F D F P L Y Y K M D E A F D
 961
 AATAACAACATTCCAGCATTAGTCTATGCCCTACAAAACGGACAACTGTAGTTTCGAGA
 N N N I P A L V Y A L Q N G Q T V V S R
 1021
 GATCCATTTAAGGCAGTAACCTTTTCGTTGCCAATCATGACACAGATATAATATGGAACAAG
 D P F K A V T F V A N H D T D I I W N K
 1081 IV
 TATCCAGCATATGCGTTCATATTGACATATGAGGGACAGCCAGTAATATTCTACAGGGAC
 Y P A Y A F I L T Y E G Q P V I F Y R D
 1141
 TTTGAGGAATGGCTGAACAAGGATAAGCTAATTAACCTCATTTGGATCCATGATCATTG
 F E E W L N K D K L I N L I W I H D H L
 1201
 GCAGGAGGAAGCACAACAATTGTCTACTACGACAACGATGAGCTCATATTTGTGAGAAAT
 A G G S T T I V Y Y D N D E L I F V R N
 1261
 GGAGATTCTAGAAGGCCTGGGCTTATAACTTACATTAACCTTGAGCCCTAACTGGGTTGGT
 G D S R R P G L I T Y I N L S P N W V G
 1321
 AGGTGGGTATACGTTCCAAAGTTTGCAGGGGCTTGTATTTCATGAATACACTGGAAACCTA
 R W V Y V P K F A G A C I H E Y T G N L
 1381
 GGAGGATGGGTAGATAAAAGAGTAGATAGTAGCGGATGGGTATACCTAGAGGCACCACT
 G G W V D K R V D S S G W V Y L E A P P
 1441
 CACGATCCAGCTAACGGCTACTATGGGTACTCCGTATGGAGTTATTGTGGTGTGGGTGA
 H D P A N G Y Y G Y S V W S Y C G V G ***
 1501
 CTTTTTCTTTTTCTTTTTTAACAATGGGAGAAGTGCAAATAC

FIG.1

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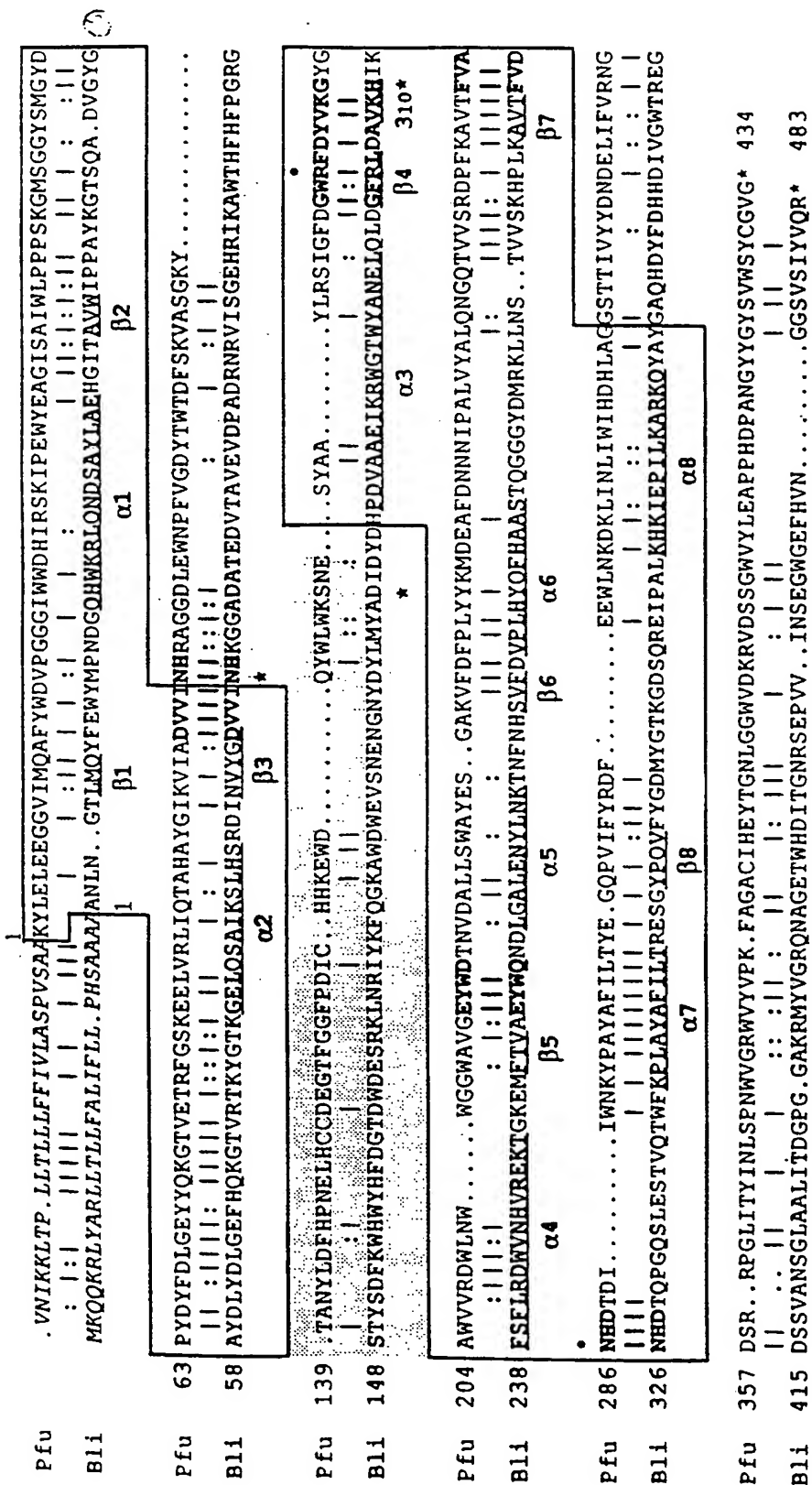


FIG. 2

FIG. 3A



FIG. 3B

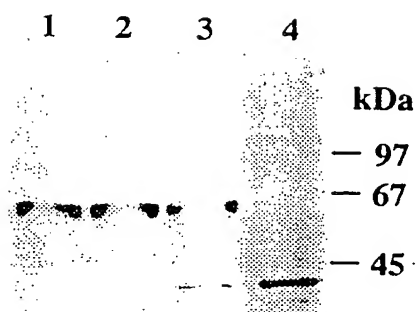


FIG. 3C



FIG. 4A

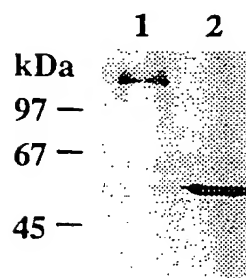


FIG. 4B



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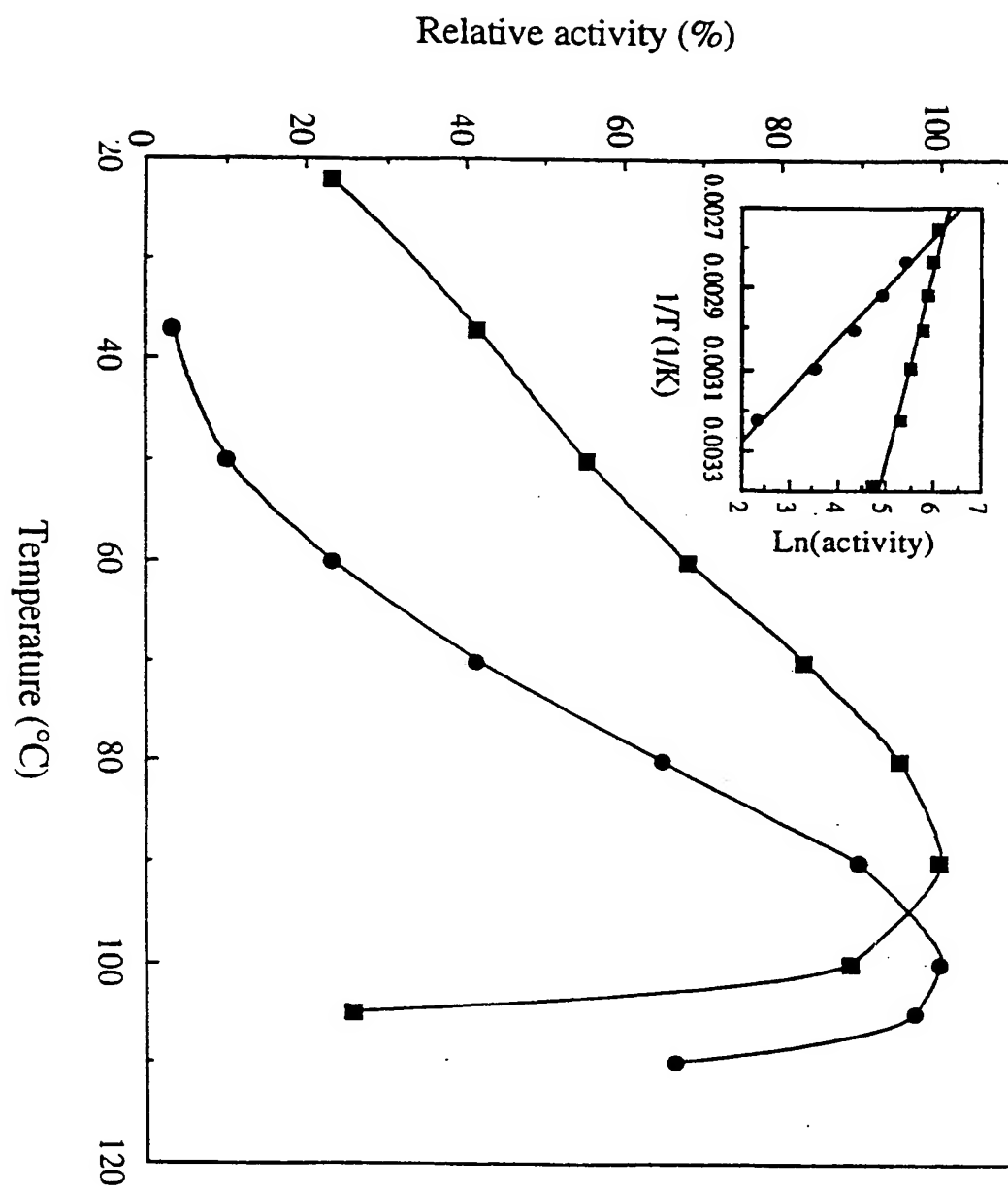


FIG. 5

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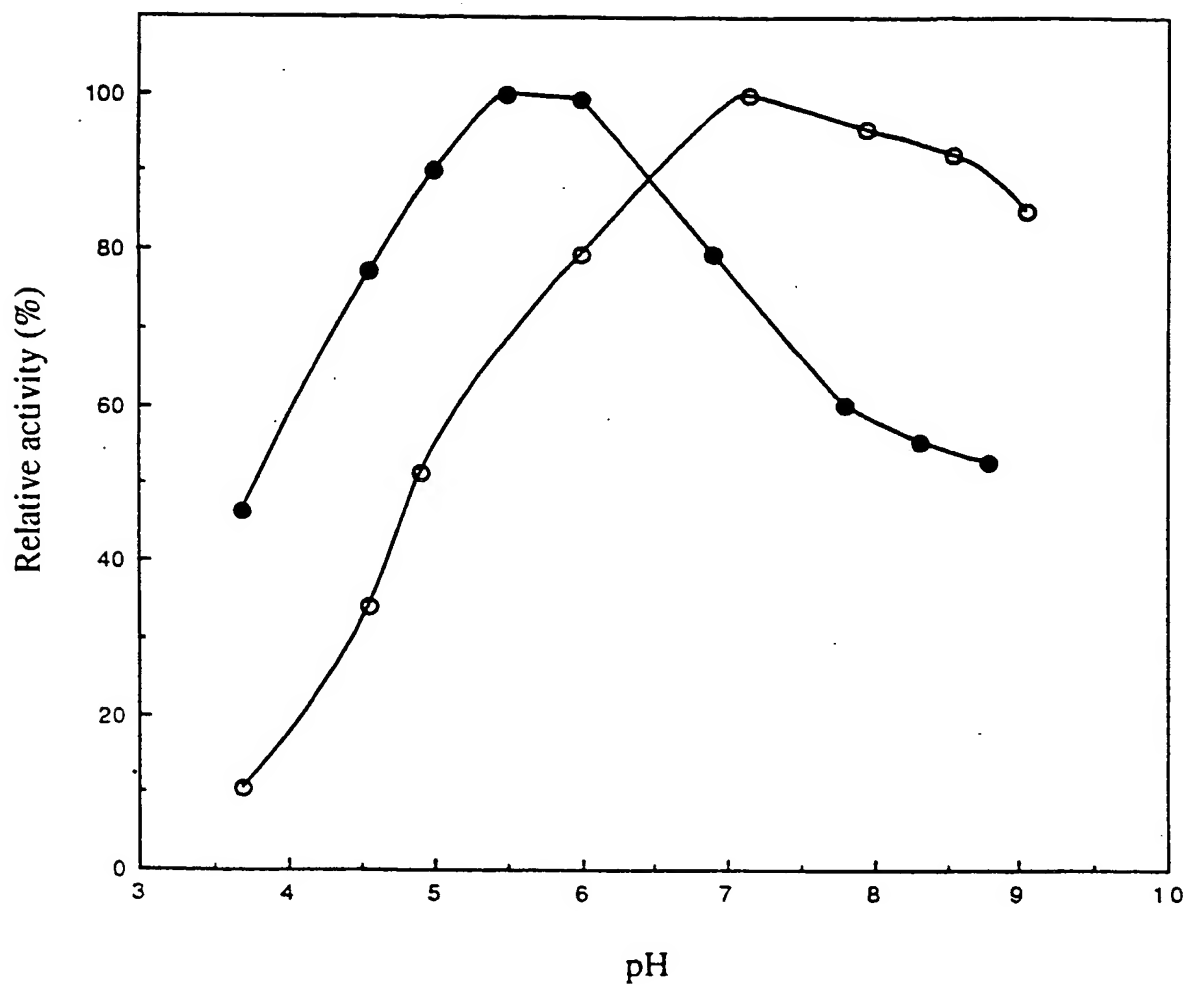


FIG. 6

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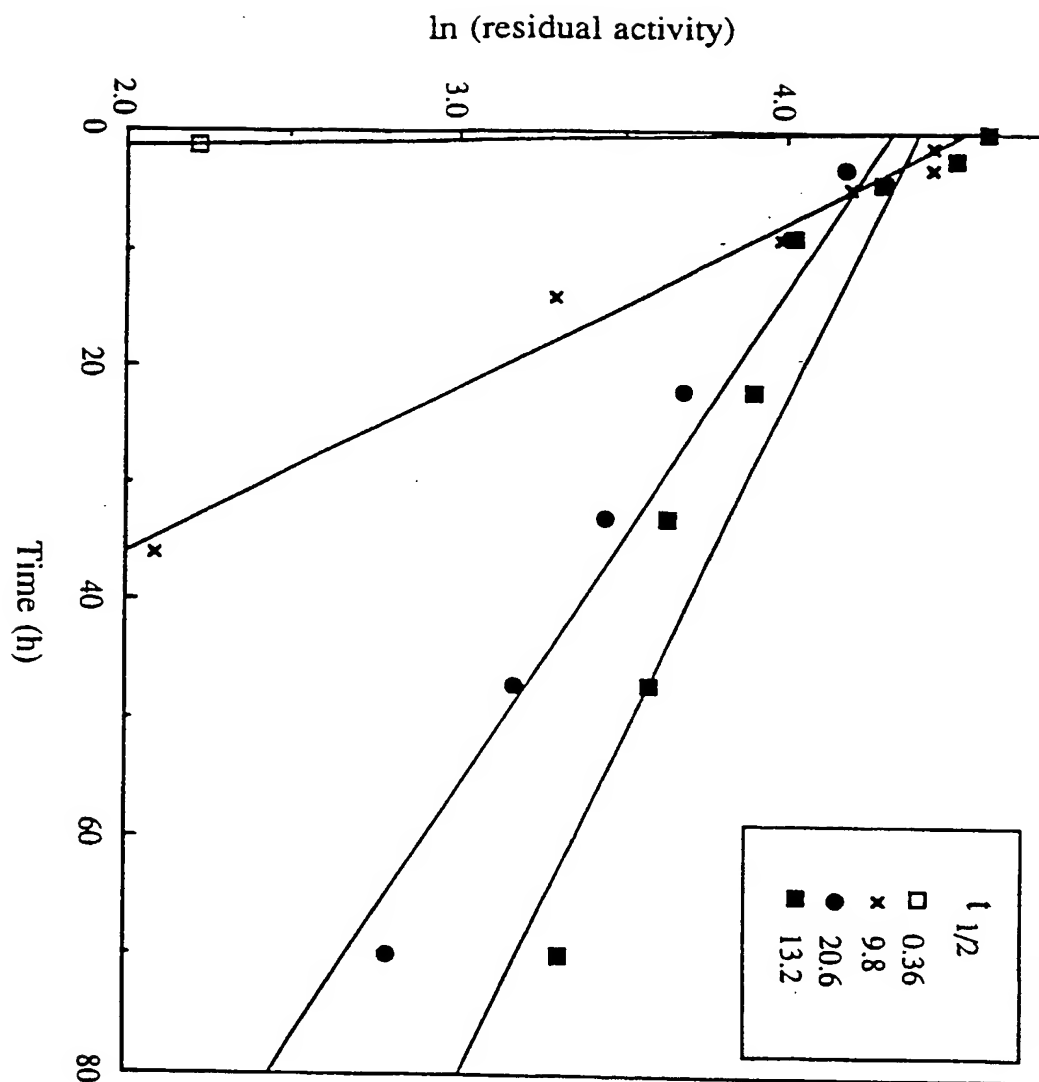


FIG. 7

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07192

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/28, 15/56

US CL :435/202; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/202; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

search terms: alpha-amylase, Pyrococcus furiosus, extracellular, secret?, thermostable

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	KOCH, et al. Extremely thermostable amylolytic enzyme from the archaeobacterium Pyrococcus furiosus. FEMS Microbiology Letters. 1990. Vol. 71. pages 21-26, see entire document.	1-11 ----- 12-18
X --- Y	LADERMAN et al. The Purification and Characterization of an Extremely Thermostable alpha-Amylase from the Hyperthermophilic Archaeobacterium Pyrococcus furiosus. The Journal of Biological Chemistry. 15 November, 1993. Vol. 268. No. 32. pages 24394-24401, see entire document.	1-11 ----- 12-18

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

25 JUNE 1998

Date of mailing of the international search report

11 AUG 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07192

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LADERMAN et al. Alpha-Amylase from the Hyperthermophilic Archaeobacterium Pyrococcus furiosus. The Journal of Biological Chemistry. 15 November 1993. Vol. 268. No. 32. pages 24402-24407, see entire document.	1-18

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